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Phylogeography of *Koramius charltonius* (Gray, 1853) (Lepidoptera: Papilionidae): a case of too many poorly circumscribed subspecies

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Abstract. *Koramius charltonius* (Gray, 1853) (Lepidoptera: Papilionidae) is distributed in the mountains of Central Asia. We analysed genetic and phylogeographic patterns throughout the western part of its range using a mitochondrial marker (COI). We also analysed the wing pattern using multivariate statistics. We found that the species contains several unique haplotypes in the west and shared haplotypes in the east. The haplotype groups do not correspond to the wing pattern and also the described subspecies do not correspond to either the haplotypes or the groups circumscribed by the wing pattern. Currently, there are more than ten subspecies of *K. charltonius* in Central Asia; based on our analyses we suggest a reduction to only five of them. The following nomenclatural changes are applied: (1) *K. charltonius aenigma* Dubatolov & Milko, 2003, **syn. n.**, *K. charltonius sochivkoi* Churkin, 2009, **syn.n.**, and *K. charltonius alrashid* Churkin & Pletnev, 2012, **syn. n.** are new synonyms of *K. charltonius romanovi* (Grum-Grshimailo, 1885); (2) *K. charltonius marusya* Churkin & Pletnev, 2012, **syn. n.**, *K. charltonius eugenia* Churkin, 2009, **syn. n.**, *K. charltonius anjuta* Stshetkin & Kaabak, 1985, **syn. n.**, and *K. charltonius mistericus* Kaabak, Sotchivko & Titov, 1996, **syn. n.** are new synonyms of *K. charltonius vaporosus* (Avinov, 1913); and (3) *K. charltonius safronovi* Korb, Shaposhnikov, Zatakovoy & Nikolaev, 2013, **syn. n.** is a new synonym of *K. charltonius voigti* (Bang-Haas, 1927).

Introduction

The systematics of Parnassiinae (Papilionidae, Lepidoptera) is complicated and has been a subject of many debates. The first classification was created by Austaut (1889) based on the sphragis structure (sphragis is a part of female genitalia, formed after copulation). The next classification was based on wing venation and was published by Stichel (1906). At the same time, other classifications based on male genitalia and wing pattern were created (Moore 1902; Sokolov 1929; Bryk 1935). This classification treated species-groups as separate genera or subgenera. The last genitalia-based classification was created by Korshunov (1988, 1990); he erected four generic-group taxa and built a new system for the subfamily. Kreuzberg and Dyakonov (1990) divided the 'genus *Parnassius*' into six separate (sub)genera (unable to decide between the two, they used this term) based on larval host-plant alkaloids. Many other modifications of the Parnassiinae classification were made based on wing pattern, genitalia structures, immature stages and/or host-plants preferences (Ford 1944; Munroe 1961; Eisner 1974; Higgins 1975; Hiura 1980; Hancock 1983; Igarashi 1984; Koçak 1989; Korb and Bolshakov 2011; etc.). Two major directions found in almost all the

proposed systems are splitting Parnassiini into several genera or placing all the taxa into a single genus *Parnassius* with several subgenera or groups.

Molecular studies in the last two decades did not change these systems in general. Yagi et al. (2001) showed that *Driopa stubbendorfii* (Ménétriès, 1849) and *D. glacialis* (Butler, 1866) descend from the same ancestor. Omoto et al. (2004) showed that *Hypermnestra* Ménétriès, 1848 is more closely related to *Parnassius* than to *Archon* Hübner, 1822, and they divided *Parnassius* into eight groups. Katoh et al. (2005) made the same conclusions. Nazari and Sperling (2007) and Nazari et al. (2007) showed that *Hypermnestra* and *Parnassius* form sister groups. They also found that the *Parnassius* ancestor originated in the Iranian Plateau and Central Asia. Michel et al. (2008) placed *Baronia* Salvin, 1893 into Parnassiinae and divided *Parnassius* into eight subgenera; the same division (but without placing *Baronia* inside *Parnassiinae*) was made by Omoto et al. (2008). Korb (2012) divided *Parnassius* into six genera based on male genitalia, host plants, and molecular data.

Using all the available data on Parnassiinae and published phylogenetic trees, we can conclude that 1) directions in trophic evolution in Parnassiinae (Kreuzberg and Dyakonov 1990) are similar to those in morphological evolution; and that 2) all morphological modifications in male genitalia are synapomorphic (i.e., every group having characteristic features is monophyletic) (these morphological groups correspond to published molecular groupings – for more details about male genitalia in Parnassiinae see Korb 2012).

Based on these conclusions, we follow the Parnassiinae classification developed by Sokolov (1929), Bryk (1935), Eisner (1974), Korshunov (1988, 1990), Korb (2012) and other authors, treating species-groups or subgenera as stand-alone genera, and we adopt the idea that the classification should be based on all available morphological and molecular data, rather than on selection of only one "taxonomically important" set of characters. Below is the list of taxa that we recognize as genera, together with their diagnostic features.

Genus *Driopa* Korshunov, 1988. Diagnostic characters: primary host-plants are Fumariaceae; saccus well developed; uncus and tegumen connection solid; gnathos present; valva without harpa, no caudal branches, no chaetae; sphragis massive, sclerotized, and occupying more than 3 abdominal tergits. In cladograms as a separate clade or sister to *Koramius* (see for example Omoto et al. 2004; Korb 2012).

Genus *Parnassius*. Diagnostic characters: primary host-plants are Crassulaceae, secondary host-plants are Fumariaceae; uncus and tegumen connection not solid; saccus reduced; valva with a massive harpa; sphragis not massive, occupying fewer than 3 abdominal tergits. In cladograms always as separate clade.

Genus *Tadumia* Moore, 1902. Diagnostic characters: host-plants are Crassulaceae or Fumariaceae; gnathos present; valva divided into sclerotised basal lobe with long harpa and non-sclerotised caudal lobe; uncus massive, sclerotised; sphragis weakly sclerotised. In cladograms always close to *Koramius*.

Genus *Kreizbergius* Korshunov, 1990. Diagnostic characters: host-plants are Scrophulariaceae; sphragis absent; uncus with only one lobe; uncus and tegumen connection solid; valva small, scale-shaped; phallus very long and curved (almost 90 degrees). In cladograms always as separate clade.

Genus *Sachaia* Korshunov, 1988. Diagnostic characters: primary host-plants are Fumariaceae; subscaphium present; uncus consisting of two separate lobes; saccus massive and with two parts; sphragis skinny. In cladograms most often clustering with *Kreizbergius*, but sometimes as separate clade (Omoto et al. 2004; Korb 2012).

Genus *Koramius* Moore, 1902. Diagnostic characters: host-plants are Fumariaceae; gnathos absent; uncus with direct connection to tegumen; harpa located in the median part of valva; valva with sets of chaetae; saccus cylindrical; sphragis tube-shaped. In cladograms always as separate clade. Divided into two subgenera: *Koramius* – with a two-lobed tube-shaped sphragis, short uncus (shorter than tegumen) and short harpa (less than half of valval length); *Kailasius* Moore, 1902 – with a one-lobe tube-shaped sphragis, long uncus (equal length or longer than tegumen) and long harpa (longer than half of valval length). See male genitalia in Figs 1–3.

***Koramius charltonius* (Gray, 1853)**

Koramius charltonius (Gray, 1853) (=*Parnassius charltonius*) is one of the most enigmatic butterfly species from the Central Asian mountains and as such it appears to be of never-ending interest to butterfly collectors. Almost every known population has been described as a standalone subspecies, making one wonder whether this is a case of taxonomic inflation, similar to what is seen in other species of the genus *Parnassius* (Rose 1995). The species belongs, according to Bryk (1935), Korshunov (1988, 1990), Korb (2012) and many other authors, to the genus *Koramius*, subgenus *Kailasius* together with *K. autocrator* (Avinov, 1910), *K. inopinatus* (Kotzsch, 1940), *K. davydovi* (Churkin, 2006), and *K. loxias* (Püngeler, 1901) (Omoto et al. 2004; Michel et al. 2008; Condamine et al. 2012; Korb 2012). All of them are distributed in the area of Central Asian mountains.

The mountains of Central Asia represent a biodiversity hotspot between rather poor arid lowlands and quite rich mountainous habitats (Kohler and Maselli 2009). The area consists of several mountain ranges (Alai, Tian Shan, Hindukush, Pamir, Karakoram and Kunlun) and is connected to the Himalayas. Central Asia experienced several cooling and aridification events since the Eocene-Oligocene Transition (cf. Zhang et al. 2014). The area was also an important crossroad between Asia via Iranian and Anatolian plateaus and the Mediterranean region (Manafzadeh et al. 2014). Finally, the mountains were essential for the ancestral distribution of many species now occurring further north (e.g., Kleckova et al. 2015) and also played an important role as refugia for northern species during glacial periods (Zhang et al. 2013). Michel et al. (2008) documented in several species of the genus *Koramius* that the mitochondrial lineages show a geographically structured latitudinal pattern.

Although there are several different species concepts, there is some agreement among practicing systematists on what constitutes a species. However, the same cannot be said for subspecies, but as the discussion about subspecies concept is not the aim of our study, we follow the one by Braby et al. (2012): "the definition of subspecies [should] be restricted to extant animal groups that comprise evolving populations representing partially isolated lineages of a species that are allopatric, phenotypically distinct, and have at least one fixed diagnosable character state, and that these character differences are (or are assumed to be) correlated with evolutionary independence according to population genetic structure". The main conclusion is simple: if it is impossible to extract even a single distinguishable feature for a whole population, this is not a distinct subspecies.

We use *Koramius charltonius* as an example of a species with many described subspecies of uncertain or unstable status. From Central Asia, subspecies have been described from almost all locations where the species was found in the last 20 years. The distribution is probably wider than known and could correspond to a distribution of its host-plants, *Corydalis stricta* Steph. & Fisch. and *C. gortschakovii* Schrenk. (Fumariaceae) (Pototski and Salo 2012).

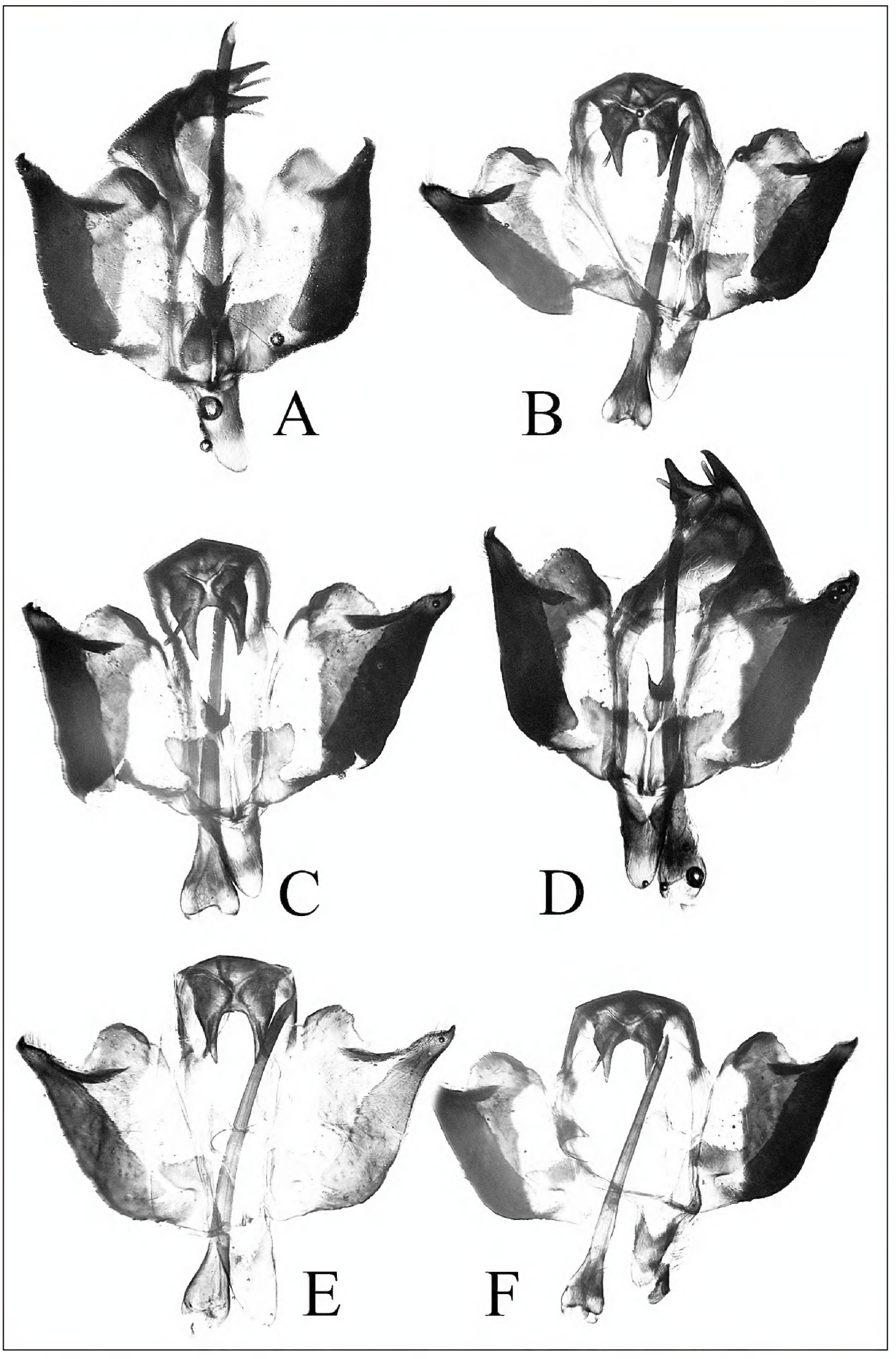

Figure 1. Male genitalia of *Driopa mnemosyne* (Linnaeus, 1758) from different locations: **A** – Kirghizia, Suusamyrtoo Mts., right shore of Suusamyr River, 2300 m; **B** – Russia, Caucasus, Teberda; **C** – EU, Slovakia, Boleraz; **D** – Kirghizia, Kirghiz Mts., Issyk-Ata Valley, 2000 m; **E** – Kirghizia, Alai Mts., Kul-Kush Valley; **F** – Kazakhstan, Transili Alatau Mts., Bolshaya Almaatinka Valley, 2500 m.

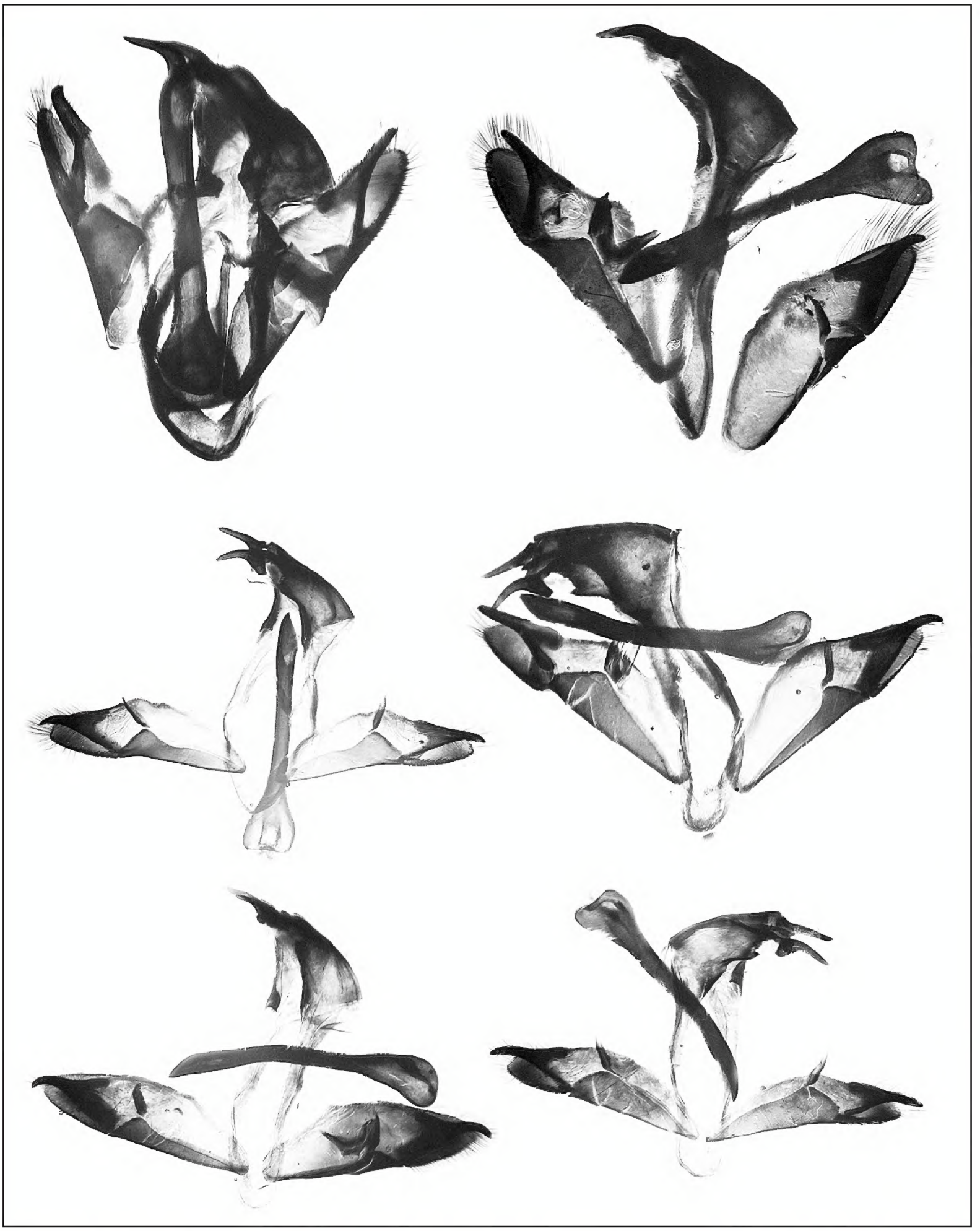

Figure 2. Male genitalia of *Parnassius apollonius* (Eversmann, 1847) from the same locality. Kirghizia, Suusamyr Valley, west shore of Karakol River, 2200–2500 m.

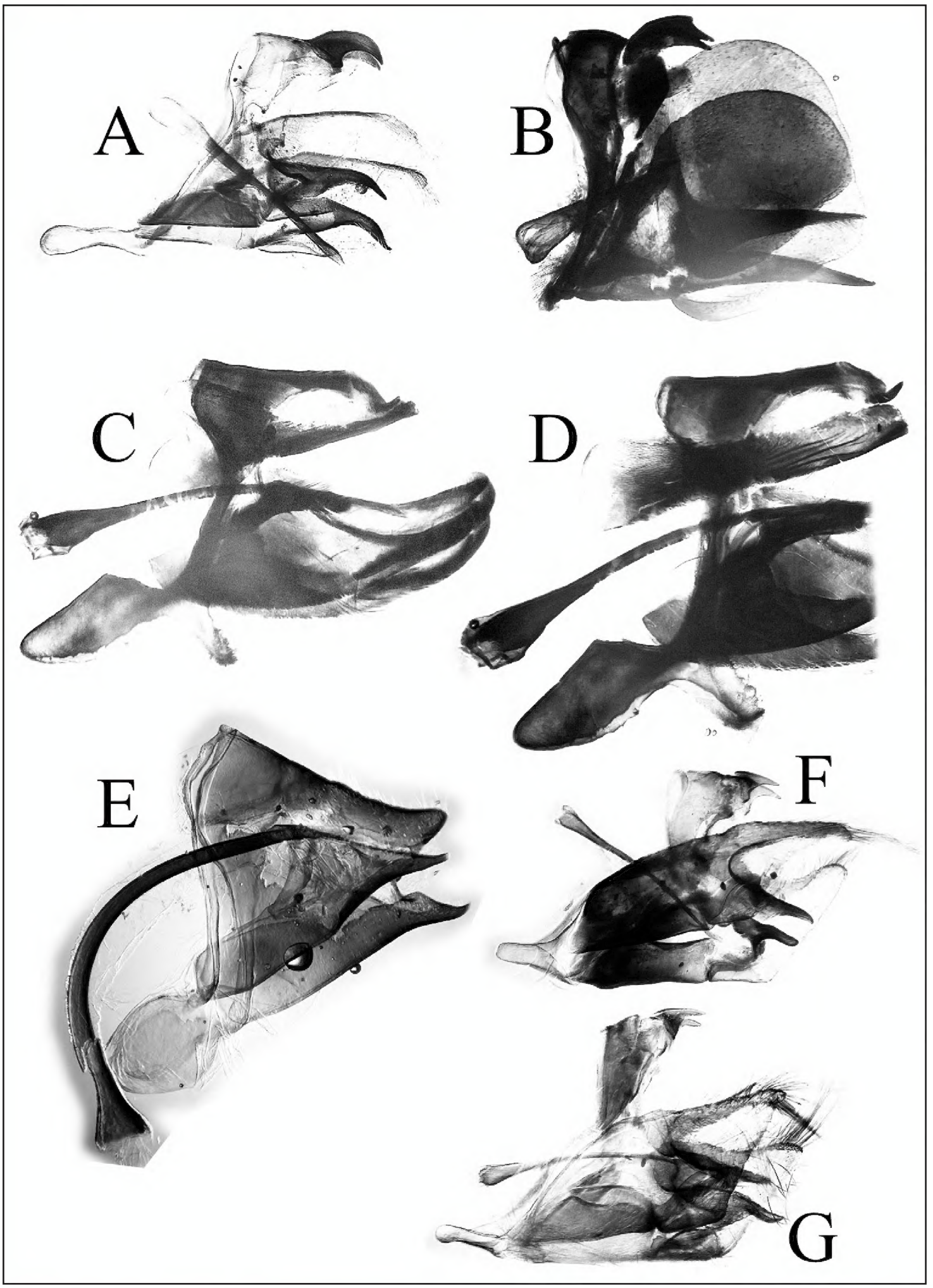

Figure 3. Male genitalia of Parnassiinae. **A** – *Koramius* (*Kailasius*) *autocrator*, Tajikistan, Sarezskoe Lake; **B** –*Lingamius szechenyii* (Frivaldsky, 1886), China, Qinhai near Tsa-Ka; **C, D** – *Sachaia tenedius* (Eversmann, 1851), Russia, Transbaikal, Mondy (in C subscaphius removed); **E** – *Kreizbergius simonius* (Staudinger, 1889), lectotype; **F** – *Koramius* (*Koramius*) *staudingeri* (Bang-Haas, 1882), lectotype; **G** – *K. stoliczkanus* (C. & R. Felder, 1865), Kashmir.

Recently Churkin and Michel (2014) sequenced a number of specimens of *K. charltonius* from various mountain ranges and they split the species into two separate species, *K. charltonius sensu stricto* and *K. romanovi* (Grum-Grshimailo, 1885). They also reassigned various subspecific names into these two species. However, we argue that their conclusions are unsupported by their own molecular data, since *K. charltonius* appears deeply paraphyletic in the published phylogenetic tree. First lineage to branch off is a sample of *K. c. voigti* (Bang-Haas, 1927) from Afghanistan, the next branch is Chinese *K. c. charltonius* (the type population!) and only after this there is a separation of *K. c. romanovi*. Also, differences in genitalia can be artefacts as proposed for instance by Stradomsky (2005). He showed that if the genitalia slide is not prepared in a uniform way of pressing the glass top onto its glass base, even specimens taken from the same population will show differences in genitalia features. In the genitalia figures by Churkin and Michel (2014), it can be seen that these micropreparations are not pressed: they have very characteristic light reflection parts which can be seen only if light falls directly on a liquid drop (without a glass top) where genitalia are located simply because the surface is not as flat as when the preparation is pressed. Therefore, in our opinion these genitalia pictures and their analysis are untrustworthy. We researched the male genitalia variability in Parnassiinae: *Driopa mnemosyne* (Linnaeus, 1758) (over 200 dissected specimens), *Parnassius apollonius* (Eversmann, 1843) (over 100 dissected specimens), *Parnassius tianschanicus* Oberthür, 1879 (over 50 dissected specimens), *Koramius delphius* (Eversmann, 1843) (over 100 dissected specimens), *Lingamius hardwickii* (Gray, 1831) (over 50 dissected specimens), as well as all other species inside this group (1–20 dissected specimens), and no variability has been found (Korb 2012). In Fig. 1 (specimens from different localities) and Fig. 2 (specimens from the same locality), this lack of variability can be seen from several examples of male genitalia.

Koramius charltonius' (Fig. 4) range includes north-western Nepal, western-most Tibet, the mountains in north-western India and northern Pakistan, north-eastern Afghanistan, Tajikistan, and southern Kyrgyzstan (Kocman 2009: 124). According to Weiss (1991), 18 subspecies of *charltonius* were recognized by the end of the 20th century. Since publishing of his book, in the last 25 years, 11 new subspecies have been added, a ratio close to one new subspecies per two years: *ljudmilae* (Lesin and Kaabak 1991), *mistericus* (Kaabak et al. 1996), *aenigma* (Dubatolov and Milko 2003), *bamianicus* (Heinkele 2003), *eugenia*, *sochivkoi*, *varvara* (Churkin 2009), *platon* (Sotchivko and Kaabak 2011), *marusya*, *alrashid* (Churkin and Pletnev 2012), *safronovi* (Korb et al. 2013). Ten of these 11 taxa have been described from the countries Tajikistan and Kyrgyzstan. Korb and Bolshakov (2011: 19–20) listed nine subspecies in these countries (taxon *sochivkoi* has been synonymised with *romanovi*; Korb and Bolshakov 2011).

Many described taxa belonging to *K. charltonius* are very difficult to place into a logical system because differences described in original descriptions begin to disappear in larger series. The wing pattern and even wing venation in Parnassiinae are very variable and cannot be used for taxon differentiation without statistical support (Korb 2012). Not even one description of a new subspecies of *K. charltonius* was based on statistically supported features (no statistical analysis based on the type or non-type series was ever made). Due to the high commercial interest, the subspecies situation is unclear and complicated, but it also gives us enough material to study a Central Asian species with fragmented distribution in high mountains suspected to have full interpopulation isolation because of the island effect. Below is the list of subspecific names of *Koramius charltonius* in Central Asia (without synonymisations). For the distribution of type localities, see Fig. 5 (numbers in Fig. 5 are taken from Table 2).

Figure 4. *Koramius charltonius* (Gray, 1853). Imago (*mistericus*, paratype, KP689312), genitalia (Chitral, Baroghil Pass), host-plant (*Corydalis gortschakovii*), habitat (Tajikistan, West Pamirs, 35–45 km N of Khorog, the type locality of *safronovi*) and sequenced specimens.

Figure 5. Distribution map of *Koramius charltonius* (Gray, 1853) in Central Asia. Black circles – type localities of described subspecies with no DNA sampled by us (mined from GenBank); black circles with grey ovals – type localities with sampled specimens (paratypes or topotypes). For numbers see Table 2.

1) *aenigma* Dubatolov & Milko, 2003. Type locality: "Kyrghyzstan, east from the Alai valley, right bank of the Kyzylsuu river at its confluence with the Koksuu river, loess-pebble bluff, 2900 m".

2) *alrashid* Churkin & Pletnev, 2012. Type locality: "NE Alai, Gulcha river".

3) *anjuta* Stshetkin & Kaabak, 1985. Type locality: "East Pamir, Mynkhadzhim mountain gorge, 4200 m".

4) *deckerti* Verity, 1907. Type locality: "Kaschmir, 4200–4800 m".

5) *eugenia* Churkin, 2009. Type locality: "Tadjikistan, Muksu R.".

6) *ljudmilae* Lesin & Kaabak, 1991. Type locality: "Tadzhikskaya SSR, Gissarsky Mts., Diakhan-Dara upper stream 40 km N of Shakhrinav, 3700 m".

7) *marusya* Churkin & Pletnev, 2012. Type locality: "Tajikistan, Obikhingou r.".

8) *mistericus* Kaabak, Sotchivko & Titov, 1996. Type locality: "Tadjikistan, South-East Pamirs, western spurs of Sarykolsky Mts Ridge, Dunkeldyk Lake, rocky crumbling slope, 4300 m above sea level".

Table 1. Material used in the morphometric analysis.

N	Subspecies	No. males	No. females	Locality
1	*aenigma*	29	8	East Aiai, Kok-Su/Kysyl-Su (Kyrgyzstan)
2	*alrashid*	13	13	East Alai, Gulcha (Kyrgyzstan)
3	*anjuta*	38	32	Mynkhadzhir (Tadjikistan)
4	*charltonius*	37	29	Tibet
5	*deckerti*	33	31	Ladakh
6	*flaugeri*	13	13	Jammu and Kashmir (India)
7	*kabiri*	15	14	Vantch Mts., Gushkhon (Tajikistan)
8	*ljudmilae*	15	12	Hissarsky Mts. (Tajikistan)
9	*marusya*	11	11	Obikhingou (Tajikistan)
10	*mistericus*	-	11	Dunkeldyk Lake env. (Tajikistan)
11	*nuristanus*	7	7	Nuristan (Afghanistan)
12	*platon*	16	15	Turkestansky Mts., Isfana (Kyrgyzstan)
13	*romanovi*	29	27	Aram-Kungei, Transalai Mts (Kyrgyzstan)
14	*safronovi*	2	7	Khorog env. (Tajikistan)
15	*sochivkoi*	26	26	North-East Alai, Ak-Bura (Kyrgyzstan)
16	*vaporosus*	42	38	Darvaz, Viskharvi Pass; Darvaz, Obimazor River (Tajikistan)
17	*varvara*	10	10	Tian-Shan, Dzhaman-Too Mts. (Kyrgyzstan)
18	*voigti*	11	11	Panjshir Valley (Afghanistan)
	In total:	**347**	**315**	

9) *platon* Sotchivko & Kaabak, 2011. Type locality: "SW Kyrgyzstan, Turkestansky Mts. Range, Sarkat River, 1500 m above s. l.".

10) *romanovi* Grum-Grshimailo, 1885. Type locality: "Transalai" (by the lectotype). Actually type locality shown in the original description located in mountainous pass near Katta-Karamuk village, South Kyrgyzstan.

11) *safronovi* Korb, Shaposhnikov, Zatakovoy & Nikolaev, 2013. Type locality: "Tajikistan, South-West Pamir, Shakhdarinsky Mts., 35–45 km N of Khorog, 3700 m".

12) *sochivkoi* Churkin, 2009. Type locality: "North-East Alai, Ak-Bura River, 2600–2700 m".

13) *vaporosus* Avinov, 1913. Type locality: "Bukhara, Darvaz, Viskharvi Pass".

14) *varvara* Churkin, 2009. Type locality: "Kyrgyzstan, Dzhaman-Too Mts., Karasu R., …2900 m".

Recently the phylogeography of several Parnassiinae species has been studied using DNA markers (Gratton and Sbordoni 2005; Gratton et al. 2006, 2008; Todisco et al. 2010, 2012). Almost all published research concerning Parnassiinae phylogeography supports the refugial theory of their dispersal across the Palaearctic Region. From these studies it is obvious that species associated with open habitats (*Parnassius apollo* (Linnaeus, 1758) and *P. corybas* Fischer von Waldheim, 1824 complex) were more widely distributed during glacial periods, whereas *Driopa mnemosyne* (Linnaeus, 1758) had a more restricted distribution during the cold phases. Our data on *K. charltonius*, which also inhabits open habitats, supports these conclusions too.

The species *K. charltonius* is currently of high commercial interest; the new subspecies are becoming valuable commodities on the market. We stress three main aims of this study: 1) to reveal the relationships of individual populations of *K. charltonius* throughout the Central Asian mountains (western part of the distribution); 2) to investigate the phylogeographic pattern in the area; and 3) to use the available information to try to test the validity of the described subspecies.

Material and methods

Specimen sampling

For this study, we selected populations known from Central Asia (we use this term to refer to the countries of Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan). No special attention to selection of phenotypes has been done; rather, we paid much more attention to the reliability of localities for our specimens than to the external features, based on the principle that if a specimen originates from the locality which is now treated as an area of a certain subspecies, it must have this subspecies' features (including the COI sequence). All sequenced specimens are depicted in Fig. 4. The species is distributed also in Afghanistan, Pakistan, Tibet and India; however, it is not easy to get fresh material from these countries. We did use several samples from these locations to see how they are connected to our delimited area and we included samples of *K. charltonius* from GenBank (mainly from publications of Omoto et al. 2004; Katoh et al. 2005; Michel et al. 2008; Churkin and Michel 2014; Tables 2, 3) and also other taxa as outgroups and for calibration points (*Hypermnestra helios* (Nickerl, 1846), *Koramius stoliczkanus* (Felder & Felder, 1865), *K. acdestis* (Grum-Grshimailo, 1891), *K. augustus* (Fruhstorfer, 1903), *K. imperator* (Oberthür, 1883), *K. inopinatus* (Kotzsch, 1940), *K. autocrator* (Avinov, 1913), *K. loxias* (Püngeler, 1901)) (Table 4). To overcome the problem with potentially incorrect collecting information for the taxonomic part of our work, in cases of uncertainty we restricted our data to the type material and we omitted material from GenBank. We also investigated the type material deposited in various collections to compare it with our available specimens. Used material originated from the following collections: collected specimens by the first author (SK); private collections of Dr L.V. Kaabak (Moscow, Russia), Mr V.V. Titov (Zheleznodorozhny, Russia), Mr Y.B. Kosarev (Nizhny Novgorod, Russia); collections of the following institutions: Zoological Museum of Moscow University (Moscow, Russia), Zoological Institute of the Russian Academy of Sciences (St. Petersburg, Russia), Finnish Natural History Museum (Helsinki, Finland), British Natural History Museum (London, UK), Museum für Naturkunde an der Humboldt-Universität zu Berlin (Berlin, Germany).

The type specimens of the following *K. charltonius* taxa have been studied: *aenigma* (paratypes, 1 ♂, 1 ♀), *anjuta* (holotype ♂, paratypes 3 ♂, 3 ♀), *charltonius* (syntype 1 ♀), *eugenia* (paratypes 2 ♂, 2 ♀), *ljudmilae* (holotype ♂), *mistericus* (holotype ♂, paratypes 3 ♂, 3 ♀), *platon* (paratypes 2 ♂, 2 ♀), *romanovi* (paralectotypes 1 ♂, 1 ♀), *safronovi* (complete type series), *sochivkoi* (paratypes 1 ♂, 1 ♀), *vaporosus* (lectotype ♂), *varvara* (paratype ♂).

Additional material studied (all topotypes): *aenigma* (12 ♂, 6 ♀), *alrashid* (2 ♂), *anjuta* (14 ♂, 6 ♀), *eugenia* (2 ♂), *ljudmilae* (2 ♂, 1 ♀), *mistericus* (32 ♂, 12 ♀), *platon* (26 ♂, 26 ♀), *romanovi* (112 ♂, 43 ♀), *sochivkoi* (12 ♂, 2 ♀), *vaporosus* (56 ♂, 42 ♀); also non-topotypic material have been studied for morphometric analysis (see Table 1 for the number of specimens and origin).

Morphology and multivariate statistics

The genitalia in Parnassiinae do not show much variability and the majority of the taxa were described using only wing pattern. Therefore we selected ten wing pattern characteristics for males and females for 18 subspecies described from Central Asia. In total, we measured 347 males and 315 females (Table 1). The wing characteristics were: (1) postdiscal belt on forewing (complete or incomplete, coded as dummy variable; PosBe); (2) colour of discal and discoidal spots (black/grey, coded as dummy; Dspo); (3) white centres in red spots on hindwing upperside (total amount;

Table 2. Sampled specimens of *K. charltonius* (see Fig. 4).

N	"Subspecies" ID	Sequence ID	GenBank Accession Number	Locality	Haplotype (#, group)
1	*anjuta*	CHAR22/14	KP689301	Tajikistan, East Pamir, Mynkhadzhir mountains, 4200 m (PARATYPE)	14, C
2	*aenigma*	CHAR004/14	KP689302	Kyrgyzstan, eastern part of Alai valley, confluence of Rivers Kok-Suu and Kyzyl-Suu, 3000 m.	15, B
3	*alrashid*	IDPARN12/14	KP689303	Kyrghyzstan, Alai Mts., Gulcha River valley, 3200 m.	17, B
4	*eugenia*	PARN222/14	KP689308	Tajikistan, NW Pamirs, Muksu River valley, 3600 m.	10, A
5	*ljudmilae*	PARNB09/14	KP689304	Tajikistan, Gissarsky Mts., Shakhrinav environs, 3700 m.	12, A
6	*mistericus*	KORB0018/12	KP689312	Tajikistan, Sarykolsky Mts., Dunkeldyk lake, 4200 m. (PARATYPE)	14, C
7	*marusya*	PARN221/14	KP689307	Tajikistan, Darvazsky Mts., Obikhingou River valley, 3700 m (PARATYPE)	9, A
8	*platon*	KORB0017/12	KP689311	Kyrgyzstan, Turkestansky Mts., Sarkat River valley, 1500 m. (PARATYPE)	26, D
9	*romanovi*	IDPARN04/14	KP689305	Kyrgyzstan, Alai Mts., Kichik-Alai gorge, Isfairamsay River valley, 3500 m.	16, B
10	*safronovi*	CHAR003/14	KP689309	Tajikistan, W. Pamirs, 35- 40 km N of Khorog, 3800 m (HOLOTYPE)	21, C
11	*sochivkoi*	CHAR012/14	KP689306	Kyrgyzstan, Alai Mts., Kichik-Alai gorge, Ak-Bura River valley, 3500 m. (PARATYPE)	17, B
12	*varvara*	KORB0016/12	KP689310	Kyrgyzstan, Dzhaman-Too Mts., Karasu River valley, 2900 m (PARATYPE)	1, A
13	*voigti*	KORB0021/12	KP689313	Afghanistan, Koh-i-Baba Mts.	29, D

WcRSpot); (4) red spots on hindwing upperside (amount; RedHWU); (5) black submarginal oval spots on hindwing upperside (amount; BSubmHWU); (6) number of violet centres in black spots on hindwing upperside (VcBSpot); (7) configuration of middle red spot on hindwing upperside (oval/rounded, as a dummy; MRedS_ov/MRedS_ro); (8) colour of middle red spot on hindwing upperside (black/grey, coded as a dummy; MRedS_black/MRedS_grey); (9) ratio of width in apical part of marginal/submarginal belt on forewing upperside (PosBelInc); and (10) suffusion of central vein and central red spot on hindwing upperside (present/absent; dummy; HWU_NoSu/HWU_BlSu). We use these characteristics as predictors for inter-subspecific differences between two taxa. For the calculations we used Canonical Correspondence Analysis, which allows testing of explanatory variables on multidimensional ordination data, and the Forward Selection approach until the variability explained by the variables cannot be improved. We ran two separate analyses for males and for females in CANOCO for Windows ver. 4.5 (Leps and Smilauer 2003) with the Monte Carlo permutation test (999 permutations).

DNA sequencing and molecular data processing

DNA extraction, PCR and sequencing were carried out according to the protocols described in Vodolazhsky and Stradomsky (2008) and Fric et al. (2014). Primers LCO and HCO were used (Folmer et al. 1994), covering the "barcode region" of the first half of the mitochondrial gene Cy-

Table 3. Samples of *K. charltonius* from GenBank used in current study.

N	"Subspecies" ID	GenBank Accession Number	Locality	Haplotype (#, group)
1	*aenigma*	KJ961627	Kyrgyzstan: Irkeshtam, Chinese Kyzyl-Su River	29, D
2	*alrashid*	KJ961626	Kyrgyzstan: Gulcha River (2200–2500 m), East Alai	28, D
3	*amabilis*	KJ961611	India: Bhaga valley, Baralacha La (4400 m)	7, A
4	*anjuta*	KJ961616	Tajikistan: Mynkhadzhyr Mts., East Pamir	1, A
5	*charltonius*	KJ961609	China: Mandhata Mt. (4800-5200 m), W. Tibet	30, -
6	*charltonius*	DQ407774	Pakistan: Satrapa Pass	22, D
7	*deckerti*	KJ961610	India: Lamayuru (3900 m), Ladakh	5, A
8	*deckerti*	KJ961613	India: S. of Khardung La (5000 m), Ladakh	3, A
9	*ducalis*	KJ961618	Pakistan: Birmoglasht (2500-4000 m), Chitral	1, A
10	*eisnerianus*	KJ961612	India: N. of Tsokar (4600 m), Ladakh	6, A
11	*ella*	KJ961617	Pakistan: Soost (3000-4000 m), Khudabaad Mts.	2, A
12	*ella*	KJ961615	Pakistan: Phakora to Naltar (2000-3200 m), Gilgit	1, A
13	*eugenia*	KJ961629	Tajikistan: Muksu River, Peter I Mts.	23, D
14	*flaugeri*	KJ961614	Pakistan: Babusar Pass	4, A
15	*kabiri*	KJ961625	Tajikistan: Gyshkhun (3000 m), Vanch Mts., W. Pamir	13, C
16	*kabiri*	KJ961624	Tajikistan: Kuh-i-Lal (3500 m), W. Pamir	14, C
17	*ljudmilae*	KJ961630	Tajikistan: Karatag River basin (3500 m), Ghissar	24, D
18	*marusya*	KJ961623	Tajikistan: Obikhingou River (2200–2400 m), Darvas	23, D
19	*platon*	KJ961621	Tajikistan: Yangiaryk (1800-1900 m)	25, D
20	*romanovi*	KJ961620	Kyrgyzstan: Aram Kungei River (3500 m), Trans-Alai	22, D
21	*sakai*	AM231451	Sonamarg, Kashmir, India	8, A
22	*sochivkoi*	KJ961631	Kyrgyzstan: Ak-Bura River (2600–2700 m), NE Alai	27, D
23	*varvara*	KJ961628	Kyrgyzstan: Karasu River (3000 m), Dzhaman-Too Mts., Tian-Shan	29, D
24	*vaporosus*	KJ961622	Tajikistan: Obimazor (3500 m), Mazorsky Mts., Darvas	19, C
25	*vaporosus*	EF473789	No data	14, C
26	*voigti*	KJ961619	Afghanistan: Panjshir valley (3500-4000 m), Parvan Prov.	11, A

tochrome Oxidase subunit I. Sequencing was partly conducted in the Biology Centre of the Czech Academy of Sciences, and partly at the Nizhny Novgorod State University.

For the sequence processing, phylogenetic analysis, and producing a timed-tree we used the software BioEdit (Hall 1999), MEGA 6 (Tamura et al. 2013), MrBayes 3.2.4 (Ronquist et al. 2012), and BEAST v. 1.8.0 (Drummond et al. 2013). Maximum Likelihood tree was computed in MEGA (ML: test of phylogeny is bootstrap method (10000 replications); Tamura-Nei model; uniform rates and use all sites; ML heuristic method – NNI; branch swap filter very strong; codons included 1st+2nd+3rd+non-coding), and Bayesian trees in MrBayes (best substitution model was selected by MrModelTest v.2.2. as GTR+G+I; we ran the program for 10,000,000 generations, with 4 chains). In BEAST we applied a molecular clock using four calibration points from Condamine et al. (2012). The haplotype network was computed in TCS 1.21 (Clement et al. 2000). We also used MEGA for a construction of a genetic distance matrix. Distance matrices based on geographical coordinates and morphological data were constructed in R i386 3.2.3. The matrices were compared for potential correlation using Mantel test in a package *ade4* with 9999 permutations. Processing

Table 4. Samples of other *Koramius* and other outgroup species from GenBank used in this study.

N	Species ID	GenBank Accession Number	Country
1	*Hesperia comma*	HQ004516	Romania
2	*Hypermnestra helios*	FJ663610	Uzbekistan
3	*Koramius acdestis*	AM231457	China
4	*K. acdestis*	DQ407760	China
5	*K. charltonius* (wrongly determined as *inopinatus*)	EF473790	Afghanistan
6	*K. inopinatus*	AM231453	Afghanistan
7	*K. autocrator*	AM231454	Afghanistan
8	*K. autocrator*	EF473788	Tajikistan
9	*K. autocrator*	DQ351029	Tajikistan
10	*K. loxias*	AM231452	Kyrgyzstan
11	*K. loxias*	EF473791	Kyrgyzstan
12	*K. augustus*	AM231456	China
13	*K. imperator*	DQ407775	China
14	*K. imperator*	EF473793	China
15	*K. acdestis*	AM231457	China

of illustrations was done in Corel Draw X4 and Photoshop CS6. Statistical calculations were made by StatSoft STATISTICA for Windows.

We ended up excluding several samples from Churkin and Michel (2014) from the final phylogenetic and haplotype analyses because after inclusion of these samples, some of them did not cluster with our samples of the same subspecies/origin (Figs 7, 8). We cannot exclude the possibility that their data are good, but there are also two alternative explanations: first, laboratory mistake, and second, locality labelling mistake. Because we suspect an error at some level, we decided to exclude these samples (KJ958545, KJ958546, KJ958547, KJ958548, KJ958549).

Results and discussion

Morphometric analyses

The majority of our measured wing characteristics poorly separated subspecies of *K. charltonius*. The only excluded variable from the final model was the colour of MRedSp in males (for single-term tests, see Table 5). The models were highly significant both for males (first canonical axis: eigenvalue=0.855, F=20.013, $p>0.001$; all axes: trace= 3.919, F=14.635, $p<0.001$) and females (first canonical axis: eigenvalue=0.877, F=16.541, $p<0.001$; all axes: trace=5.181, F=13.327, $p<0.001$). Wing pattern elements explain 26.13% of variability between subspecies in males and 30.48% of variability in females. Males of *varvara*, *safronovi* and *flaugeri* form a cluster, characterized by round MredS; another cluster of males of *kabiri*, *deckerti*, *anjuta* and *voigti* is characterised by PosBeCom, PosBelIn and VcBSpot. Males of *alrashid*, *platon*, *aenigma*, *sochivkoi*, *romanovi*, *marusya*, and to some extent also *charltonius*, are characterised by HWU_NoSu and WcRSpot (Fig. 6A). A very different pattern is seen in females, where none of the male clusters are found (Fig. 6B). One female group is formed by *anjuta*, *safronovi* and *mistericus* and is characterised by grey MredS. Another group is formed by *alrashid*, *charltonius*, and to some extent also by

Table 5. Single term tests of wing pattern analyses for males and females of *K. charltonius* from CCA with 999 Monte Carlo permutations.

	Males		Females	
	F	**p**	**F**	**p**
PosBeCom	14.67	0.001	10.12	0.001
PosBeInc	14.67	0.001	10.12	0.001
Dspo_black	0.82	0.589	15.26	0.001
Dspo_gray	0.82	0.589	15.26	0.001
WcRSpot	11.87	0.001	14.49	0.001
RedHWU	15.81	0.001	12.92	0.001
BSubmHWU	3.43	0.002	4.17	0.001
VcBSpot	12.18	0.001	13.66	0.001
MRedS_ov	16.44	0.001	13.91	0.001
MRedS_round	16.44	0.001	13.91	0.001
MRedS_black	NA	NA	8.95	0.001
MRedS_grey	NA	NA	8.95	0.001
PosBelInc	18.55	0.001	16.04	0.001
HWU_NoSuff	12.33	0.001	4.13	0.001
HWU_BlSuff	12.33	0.001	4.13	0.001

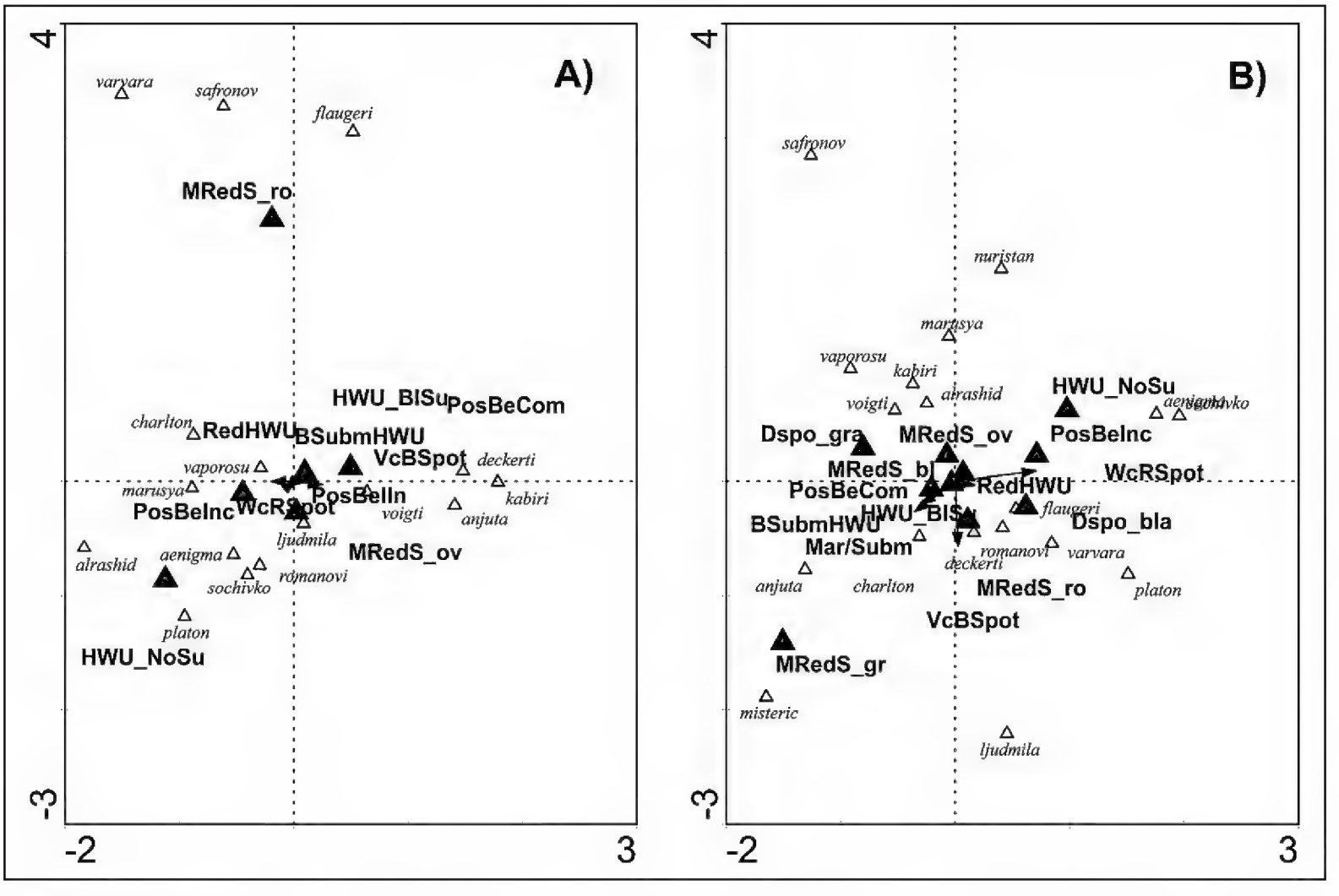

Figure 6. Ordination plots of CCA analyses of variables of wing pattern elements on subspecies of *K. charltonius*. **A**) males, **B**) females. Plotted are ordination axes 1 and 2. Continuous variables are plotted as arrows, dummy variables as white triangles. Black triangles represent the centroid of subspecies.

ljudmila and *vaporosus*, and they are characterised by oval MredS and higher ratio between Mar/Subm. Females of *aenigma*, *platon*, *flaugeri*, *varvara* and *sochivkoi* have round MredS and, together with *ljudmila*, *romanovi* and *nuristanus* also black Dspo, PosBeInc and WcRSpot.

Phylogeny and phylogeography

According to our molecular analyses (Fig. 7), *Koramius charltonius* samples form a clade that is sister to *Koramius inopinatus* + (*K. autocrator* + *K. loxias*). A surprise is that one specimen of *K. inopinatus* (Genbank Accession Number EF473790, from Omoto et al. 2004) goes inside of *charltonius* (potential causes are introgression, hybridization, or misidentification), while the second specimen of this species groups with *K. autocrator* + *K. loxias* (Fig. 7).

K. ch. charltonius from Tibet, i.e. the nominotypical subspecies, is sister to all the other populations of *K. charltonius*, which are split into two clades (ML tree; this is also supported by the network analysis and thus we prefer this arrangement), or with *K. ch. voigti* sister to other Central Asian populations (BI tree). With this exception, the BI and ML trees are very similar despite low support for the main clades. One clade is formed by populations from east Tajikistan and Kashmir (haplotype group A in Fig. 7; ssp. *ljudmilae*, *sakai*, *eugenia* and a paratype of *marusya*) plus surprisingly *varvara* from an isolated area in Kyrgyzstan. The next clade is formed by populations from Kyrgyzstan (Alai and Turkestansky Mts.) (haplotype group B in Fig. 7; *platon*, *alrashid*, *sochivkoi* and *romanovi*) as well as by close and genetically indistinguishable populations from the northern "main" mountain ranges of the Central Asian mountains, from Pamir to Tibet (haplotype groups C and D in Fig. 7; *voigti*, *safronovi*, *mistericus*, *vaporosus*, *anjuta*, *aenigma* and *charltonius s.str.*). Only populations from the eastern part of the species' range differ from the other populations. Multiple specimens from the same subspecies cluster with specimens of different subspecies in *alrashid*, *romanovi*, *vaporosus* etc. In *ljudmilae*, *voigti* and *charltonius s.str.* different specimens even belong to different clades.

Haplotype network analysis shows high haplotype divergence (Fig. 8). In total there are 30 haplotypes, which can be separated into several clusters. There are distant connections between southern (Tajikistan + Kashmir) and Kyrgyz populations and much shorter connections in haplotype rich northern and north-eastern populations. The first offshoot of *K. charltonius* on the phylogenetic trees, *K. ch. charltonius*, with our default settings does not belong to the same network. First haplotype group (A) is widely distributed across the species range. The second haplotype group is restricted to southern Kyrgyzstan. The third group is distributed in Tajikistan and Afghanistan, whereas the fourth group is again shared between a vast part of the species area.

The messiness of the phylogenetic relationships among these putative taxa is reflected in the messiness of the systematics of this species. Clearly, using the phylogenetic tree, it is impossible to circumscribe separate subspecies and this result supports our conclusions about the unrealistic number of subspecies of *K. charltonius*. On the other hand, the haplotype network visualizes the grouping and connections between the populations and it can be used for taxonomic interpretations, as it was done recently, for example, by Pazhenkova et al. (2015).

Taxonomic implications

Even when we take into account the differences between ML and BI trees, we see that the number of described subspecies is not realistic. Moreover, our results do not support the traditional division of described subspecies into groups (*sensu* Weiss 1991) as all the clades are a mix of several

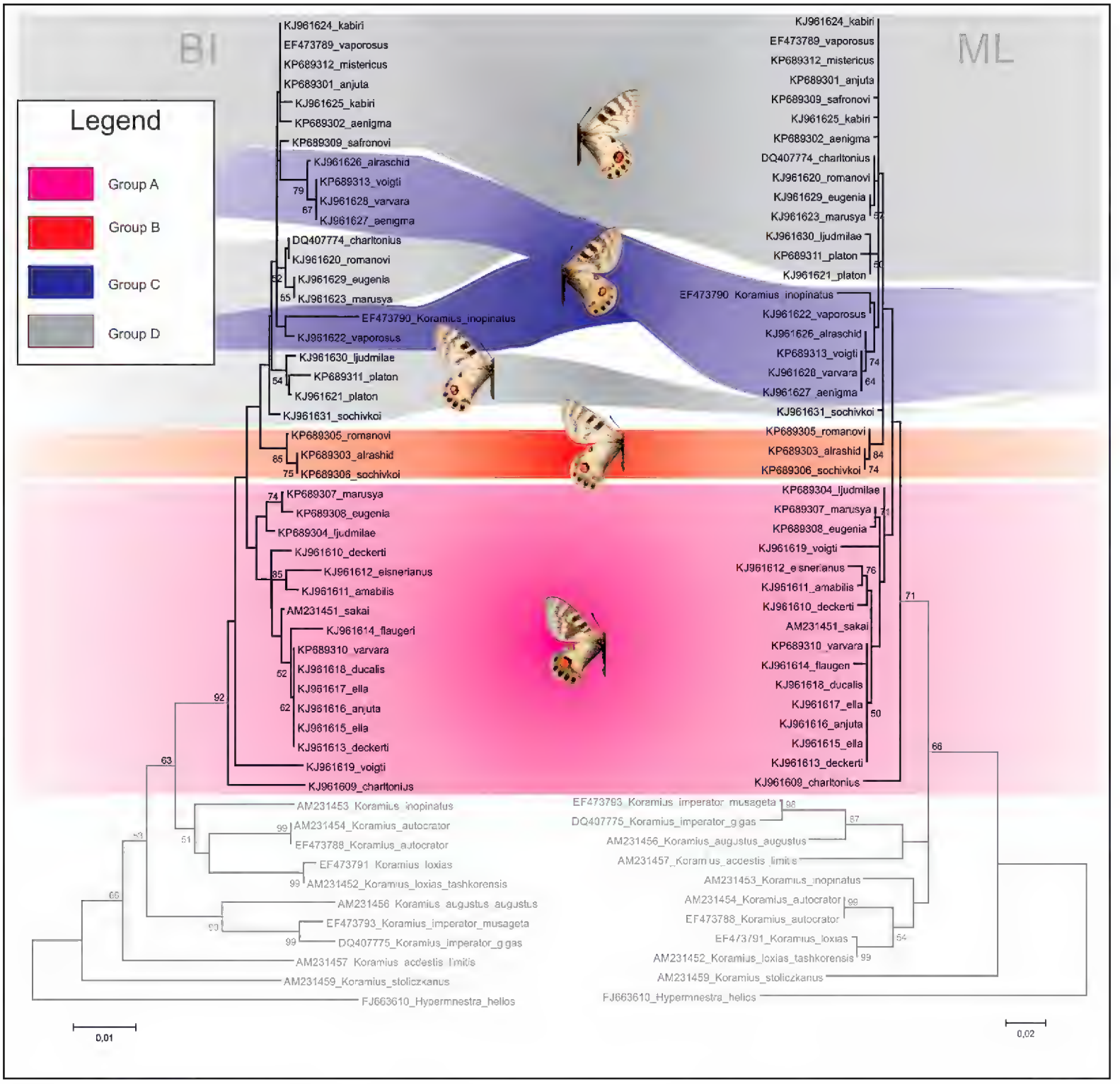

Figure 7. The Bayesian inference (BI) and maximum likelihood (ML) trees of *Koramius charltonius* and closely related taxa. Outgroups are shown in grey. The colours represent the four haplotype lineages.

subspecies groups and these groups are scattered across the tree. On the other hand, the subspecies form several clusters according to the geography (see the type localities of all subspecies of *K. charltonius* described from Central Asia in Fig. 5) as well as genetic clusters (Fig. 7), but the genetic differences inside the clusters are very small, with over 50% of sequenced specimens having a barcode pairwise distance smaller than 0.2% between them. The overall mean distance between samples was only 0.00141. Unfortunately, because of the low number of individuals per subspecies/population, we are unable to see the local variation, but evidently, while some local populations (subspecies) are well separated, other samples show that about half of described subspecies are paraphyletic, and represent local variability between closely related populations and not separate forms.

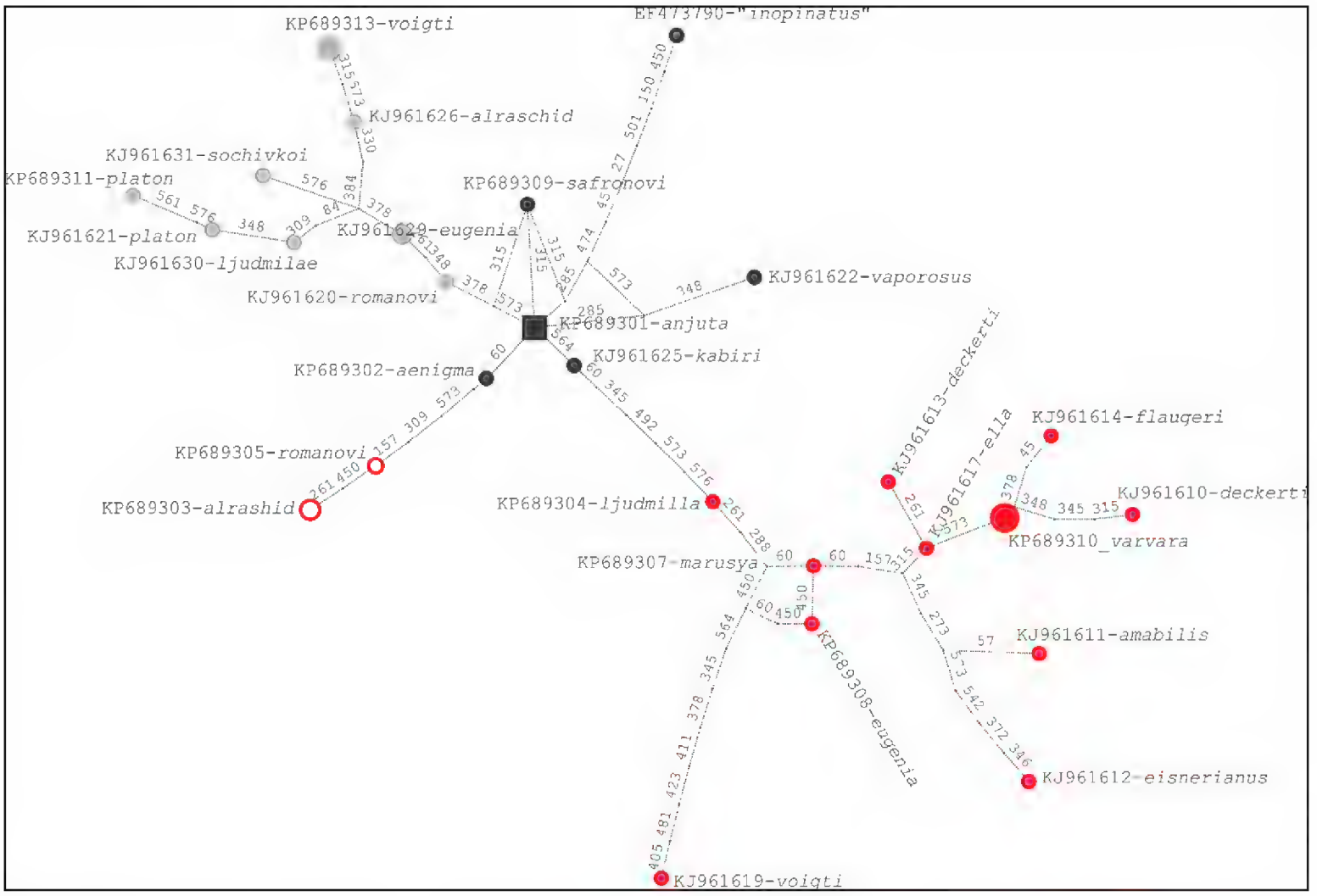

Figure 8. The haplotypes network of *Koramius charltonius* in Central Asia, median-joining, constructed using only verified sequences. Haplotype group A – red dots; group B – red circles; group C – black dots; group D – grey dots. See text for details.

The genetic distances were marginally positively correlated with geographic distances (Mantel test, r = 0.271, p = 0.071), but they were not correlated with morphological distances (r = 0.051, p = 0.324). Even when we made comparison with only males (r = -0.021, p = 0.567) or females (r = 0.082, p = 0.253), we did not find any correlation. Also, when comparing morphology distance matrices for males and females, we did not find a correlation (r = -0,001, p = 0.481). Thus the differences between these subspecies are not real; for some specimens it is not possible to identify to which subspecies of *K. charltonius* they belong without having geographical data. Recognizable phenotypes are present only in subspecies *romanovi*, *varvara*, and *platon*. Additionally, it is possible to identify some groups of subspecies according to the wing pattern.

Based on the results of our examinations, we propose a preliminary subspecific division of *K. charltonius* in Central Asia. It is possible that it will be necessary to further reduce the number of subspecies in the future because there is no clear pattern in phylogenetic trees. We used the haplotype network and morphometric data as a basis for the following division:

a) *varvara*, characterised by island-distribution in the Inner Tian-Shan (it is possible that this subspecies name will be synonymized after further investigation).

b) *romanovi*, characterized by distribution in the Alai and Transalai Mts. The southern border of this subspecies is on the high-mountainous plain starting from the southern slope of the

Transalai Mts., the eastern border is at the beginning of the Zeravshan River Valley. Synonyms: *aenigma* Dubatolov & Milko, 2003, **syn. n.**, *sochivkoi* Churkin, 2009, *alrashid* Churkin & Pletnev, 2012, **syn. n.**

c) *platon*, characterized by distribution in the north-eastern limit of the species range in Central Asia (Turkestansky Mts.) and by the mid-altitude mountainous vertical distribution (1500–2100 m). This kind of vertical distribution is unusual for this species and makes a notable ecological isolation from other known populations.

d) *ljudmilae*, characterized by the distribution in the south-eastern limit of the species distribution in Central Asia (Hissarsky Mts.). There are long distances (over 200 km) to its other closest known populations.

e) *vaporosus*, characterized by distribution in east, central and north-west Pamir and Darvaz. The borders of this subspecies are Alichur River in the south, highland plains of East Pamir in the east, the high-mountainous plain starting from the southern slope of Transalai Mts. in the north, and the eastern slopes of Darvaz Mts. in the west. Synonyms: *marusya* Churkin & Pletnev, 2012, **syn. n.**, *eugenia* Churkin, 2009, **syn. n.**, *anjuta* Stshetkin & Kaabak, 1985, **syn. n.**, *mistericus* Kaabak, Sotchivko & Titov, 1996, **syn. n.**

f) *voigti*, distributed in West Pamir and Afghan Badakhshan. The northern border is in the Akademii Nauk Mts. and adjacent glaciers. Synonym: *safronovi* Korb, Shaposhnikov, Zatakovoy & Nikolaev, 2013, **syn. n.**

Phylogeography

We present the phylogenetic relationships of populations of *Koramius charltonius* based on one mitochondrial gene from the western part of its range. Whereas two haplotype groups (A and D) are widely distributed throughout the range, two other haplotype groups (B and C) indicate a separation of larger historical areas by vicariance, first in isolated sections of the westernmost parts of the area (isolation of *platon* and *ljudmilae*), then separation of *voigti*, and then separation of the southern (*vaporosus*) and northern (*romanovi*) branches with *deckerti* in between. Shatravin (2000) summarized the history of the Pleistocene glaciation in the Pamirs and Tian-Shan (mountainous Central Asia), which consisted of three parts: first Pleistocene stage (about 74,000–70,000 years ago), second Pleistocene stage (about 54,000–52,000 years ago), and third Pleistocene stage (about 24,000–22,000 years ago).

The observed pattern suggests that the species had a wide distribution in Central Asia in the past, at least up to the southern ridges of Tian-Shan (probably wider). The first Pleistocene stage divided the area of the *K. charltonius* ancestor into several large areas separated by high mountain ridges, which were covered by glaciers: e.g., Alai and Transalai, Sary-Kol etc. (Shatravin 2000). Further separations followed in the second and third stages and inter-stage times. This is supported by larger differences between subspecies: the most ancient ones are located in the northern parts of the species area and have the COI-differences of about 1% (*varvara*, *platon*).

These results indicate a shrinking of the original range, with western populations becoming isolated due to the gradual drying of the mountains rather than dispersal. Also, it is evident that the eastern populations are not really genetically isolated and thus the majority of the described subspecies lack genetic justification.

Conclusions

We found very weak support for the traditional division (Weiss 1991) of *Koramius charltonius* into subspecies and even less into the subspecies groups; the phylogenetic division follows latitudinal geographic structure and not the formal taxonomy. We are aware of the limitations of mitochondrial data for answering questions about evolution, but our results are probably meaningful since they are concordant with geography. Also, our findings are similar to the findings of Michel et al. (2008), who, however, worked on the specific level, whereas we focused more deeply on the populations.

Many of the new subspecies descriptions are frequently inadequately erected, based rather on partial data than on systematic comparison of large series and using adequate analyses. Even worse, many of such subspecies might have been established only to increase the price of the insect specimens on the market. However, these taxa are still valid in the sense of the zoological nomenclature. Taxonomists must acknowledge every described taxon, they must place it in its correct position or synonymize it, and we have done our best with this difficult case based on all the currently available data.

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